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PRINCIPAL INVESTIGATOR: Fayth K. Yoshimura, Ph.D.

CONTRACTING ORGANIZATION: Wayne State University
Detroit, MI 48201

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Fayth K. Yoshimura

E-Mail: fyoshi@med.wayne.edu

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14. ABSTRACT

The main objective of this research project is to test the hypothesis that a newly discovered retrovirus, XMRV, is involved in the development of human breast cancer. To accomplish this, we have examined XMRV infection in breast cancer tissue in comparison with normal breast tissue. Assay conditions for the detection of XMRV protein in formalin-fixed and paraffin-embedded (FF-PE) sections by immunohistochemistry (IHC) were optimized by the identification of a monoclonal antibody (mAb 83A25) that is at least four-fold more sensitive than other antibodies used in previous studies. For this purpose we produced a cell line that expresses infectious XMRV virus by starting with plasmid DNA for the cloned XMRV genome. This cell line was also used as positive control for IHC tissue analysis. Our IHC analysis of de-identified FF-PE specimens for 28 breast cancer samples and 5 normal breast tissues have indicated that 20% of breast cancer and none of the normal breast samples express a cellular protein that is detectable with mAb 83A25, which reacts with various murine gammaretroviral envelope (Env) proteins. An amino acid search of the human genome for proteins that are closely related to these Envs identified eight different class I human endogenous retroviruses (HERVs) with sequence identities ranging from 43 to 51%. These observations suggest that the cellular protein that we have detected is a class I HERV Env, which may be associated with breast cancer. Our studies have resulted in the detection of a potentially novel breast cancer-associated protein.

15. SUBJECT TERMS
XMRV retrovirus, FF-PE breast cancer and normal breast tissues, 83A25 mAb, immunohistochemistry (IHC), human endogenous retrovirus (HERV).

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INTRODUCTION: A novel retrovirus, xenotropic murine leukemia virus-related virus (XMRV), has been linked to prostate cancer (1-2). XMRV was detectable in 23% of prostate cancer patients (2) and 4% of healthy controls (3). To determine whether XMRV is involved in the pathogenesis of other types of human cancer, such as breast cancer, we proposed to examine breast cancer specimens for the presence of XMRV. We proposed to initially examine formalin-fixed and paraffin-embedded (FF-PE) breast cancer tissue for the presence of XMRV protein by immunohistochemistry (IHC) analysis. XMRV nucleic acid would be detected to confirm results from viral protein analysis. Statistical analysis indicated that the detection of XMRV-positive cancers at a rate of 16% or greater would require 28 patient samples, assuming a 4% incidence rate in the general population (3), alpha = 0.05 (1-sided) and power = 0.80. A demonstration that an infectious retrovirus is involved in the development of breast cancer could potentially lead to the development of a new biomarker for detection, diagnosis, and prognosis. It would further provide new forms of treatment and ultimately prevention of this deadly disease, which is the leading cause of cancer death among Hispanic women and second for women of all other races.

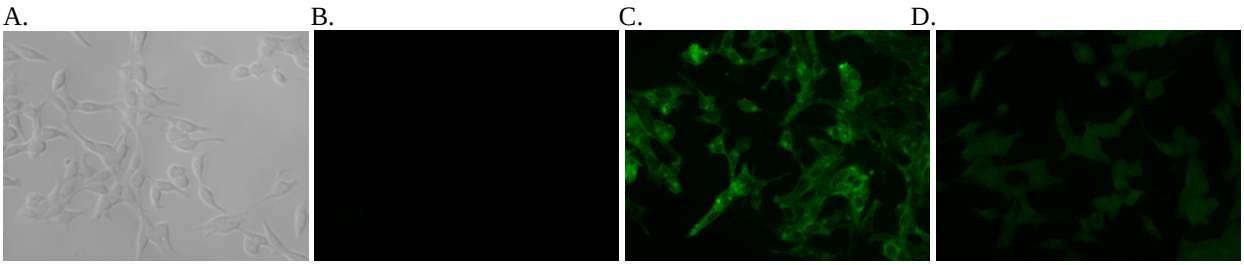
BODY: To determine whether XMRV is associated with human breast cancer, we initially examined formalin-fixed and paraffin-embedded (FF-PE) specimens for 28 breast cancer tissues and 5 normal breast tissues for the presence of viral protein as described in Task 1a. Immunohistochemistry (IHC) was used to detect XMRV envelope (Env) protein in FF-PE sections using a rat monoclonal antibody (mAb) 83A25 that recognizes the Env of murine retroviruses closely related to XMRV (4). To optimize conditions for performing IHC assays, we first generated a cell line that was infected with XMRV, which could also be used as an essential control for IHC assays of tissues. The production of virus-infected cells first required the preparation of infectious virus. This was accomplished by transfecting human 293T cells with plasmid DNA containing a clone of the XMRV genome, which was obtained from Dr. Robert Silverman (The Cleveland Clinic, Cleveland, OH) (5). Transfection was performed by treating cells with Lipofectamine 2000 (Invitrogen). Cell-free supernatant was collected and used to infect cultured human LNCaP cells. These cells were chosen because they are highly susceptible to XMRV infection (2).

To detect XMRV-infected LNCaP cells, we used an indirect immunofluorescence assay that we have used in our previous studies of other types of murine leukemia viruses (MLVs) (6). Successful XMRV Env detection was obtained with mAb 83A25 (Fig. 1). We subsequently compared the sensitivity of this antibody with a rat anti-SFFV mAb that recognizes the XMRV Gag protein and has been used to detect XMRV in previous studies by other laboratories (1). Our results showed that the 83A25 mAb was at least 4-fold more sensitive than the anti-SFFV mAb (Fig. 1). Briefly, this assay involved the growth of uninfected or XMRV-infected LNCaP cells on an 8-well chamber slide coated with poly-L-lysine, followed by incubation with either 83A25 or anti-SFFV mAb. After rinsing, cells were treated with a goat anti-rat fluorescent antibody (Alexa Fluor 488, Invitrogen). Fluorescence quantification was performed with a Zeiss Axioplan 2 microscope and Metamorph software provided by the Karmanos Cancer Institute Microscopy, Imaging and Cytometry Resources (KCI MICR) Core facility. Based on our results, we have chosen to use the 83A25 mAb for IHC tissue analysis.

As controls for IHC assays of FF-PE breast cancer tissues, we have used FF-PE sections containing uninfected or XMRV-infected LNCaP cells. For this purpose, FF-PE sections of

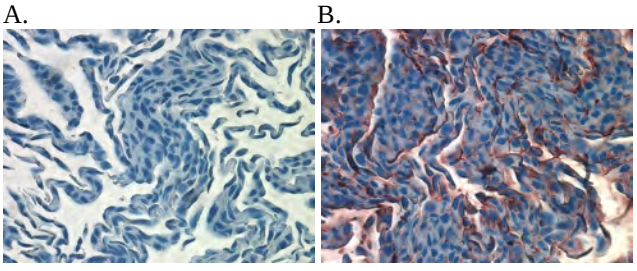
these cultured cells were prepared by following standard procedures (7). To initiate IHC studies, we have optimized conditions for these assays using the 83A25 mAb as the primary antibody. 5-µm sections were cut and placed on electrically-charged glass slides and dried at 56°C for 30 min. Sections were deparaffinized in xylene and rehydrated in decreasing alcohol concentrations (100%, 95%, 85%, 3 min each). Antigen retrieval was performed via steam treatment for 20 min in 1mM EDTA. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min. Sections were incubated with 83A25 hybridoma supernatant overnight at 4°C. After washing with PBS 3x at room temperature (RT), sections were incubated with a secondary anti-rat biotinylated antibody (Ventana Medical Systems) for 30 min at RT, and treated with a streptavidin-horseradish peroxidase conjugate (ABC) reagent. Viral protein was detected with a 3-amino-9-ethylcarbazole (AEC) chromogen reaction. Sections were counterstained with hematoxylin for 2 min at RT and dehydrated with graded alcohols. Slides were sealed with Cytoseal 60 mounting media (Thermo Scientific). FF-PE uninfected and XMRV-infected LNCaP cells that were analyzed by IHC are shown in Fig. 2.

Fig. 1. Identification of a monoclonal antibody for the detection of XMRV infection.



Comparison between 83A25 and anti-SFFV mAbs for the detection of XMRV protein by indirect immunofluorescence. (A) Bright field microscopy of XMRV-infected LNCaP cells at 200x magnification using a Zeiss Axioplan 2 fluorescence microscope (KCI MICR Core). Fluorescence images of XMRV-infected LNCaP cells treated with either (B) secondary goat anti-rat fluorescent (Alexa Fluor 488) Ab and no primary mAb; (C) primary 83A25 mAb and secondary fluorescent Ab; or (D) primary anti-SFFV mAb and secondary fluorescent Ab.

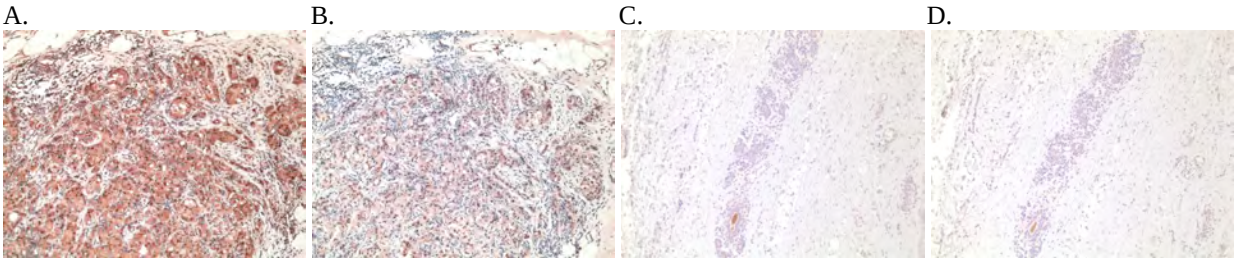
Fig. 2. Detection of XMRV infection of LNCaP cells by IHC analysis.



(A) Uninfected and (B) XMRV-infected LNCaP cells were formalin-fixed and paraffin-embedded. 5-µm sections were cut and subjected to IHC analysis with 83A25 mAb and the Ventana BASIC AEC Detection kit for horseradish peroxidase activity. Sections were counterstained with hematoxylin revealing blue nuclei. Photomicrographs were taken at 400x magnification with a Zeiss Axiophot microscope (KCI MICR Core).

Using the optimized IHC conditions described above, we proceeded to analyze de-identified FF-PE samples for 28 breast cancer and 5 normal breast tissues. Our results indicated that 20% of breast cancer specimens and none of the normal breast tissues expressed a protein that reacted with mAb 83A25 (Fig. 3).

Fig. 3. Detection of a cellular protein expressed in breast cancer tissue by IHC analysis.



IHC analysis of a breast cancer specimen (A-B) and normal tissue (C-D) was performed as described for Fig. 2. (A) Typical example of tumor cells detectable with mAb 83A25 in a breast cancer section. (B) Serial section of the same breast cancer specimen shown in (A) was treated with an isotype control (rat IgG2a anti-KLH, Abcam, Inc., Cambridge, MA). (C) Normal breast tissue treated with mAb 83A25. (D) Serial section of normal breast tissue shown in (C) that was treated with isotype control.

While we were performing this work, however, other laboratories demonstrated that XMRV arose from recombinational events in mice that were inoculated with prostate tumor cells and is not of human origin (8). Apparently, XMRV subsequently infected other human prostate cancer cell lines that were carried in culture. Moreover, it was demonstrated that XMRV-related sequences were detectable in human tissues by sensitive PCR assays because of contamination by miniscule amounts of mouse cellular DNA (9). These observations have led to the general conclusion that the detection of XMRV and XMRV-related sequences in human samples was due to artifactual reasons. Because of these results from other laboratories, we did not proceed to further analyze XMRV nucleic acid sequences in breast cancer tissues (Task 1b) nor to test the possibility that XMRV infection may be associated with breast cancer stage and grade (Task 1c).

We were, nevertheless, intrigued by our IHC results, which suggested that some breast cancer tissues express a cellular protein that is detectable with an antibody that recognizes the Env protein of various murine gammaretroviruses (4). Because of its cross-reactivity with mAb 83A25, we presumed that this cellular protein shares close amino acid homology with murine gammaretroviral Envs. To examine this possibility, we performed an amino acid search of the human genome for proteins that are closely related to different murine gammaretroviral Envs that are known to react with mAb 83A25. This search identified Env proteins of eight different class I human endogenous retroviruses (HERVs F(c)1, F(c)2, FRD, H, R(b), R/env3, T, and W) with sequence identities ranging from 43 to 51%, which is equivalent to the range of percentage identity obtained when Env sequences between the eight class I HERVs themselves are compared. This result is not surprising because phylogenetic studies have determined that class I HERVs are also gammaretroviruses, which share close homology in their *env* gene among family members of different species (10). These observations, therefore, suggest that the cellular protein that we have detected is a class I HERV Env, which may be associated with breast

cancer. This idea is strongly supported by the observation by Frank et al. (11) that class I HERV-T has increased levels of *env* transcripts in malignant human breast tissue in comparison with normal tissue. Our work, therefore, has resulted in the detection of a potentially novel breast cancer-associated protein.

KEY RESEARCH ACCOMPLISHMENTS:

- Infectious XMRV was prepared by transfecting human 293T cells with plasmid DNA containing a clone of the XMRV genome (obtained from Robert Silverman, The Cleveland Clinic, 5). Cell-free supernatant containing infectious virus was collected.
- XMRV-infected LNCaP cells were detected with an indirect immunofluorescence assay with the 83A25 monoclonal antibody (mAb) that recognizes the glycoprotein of various types of xenotropic murine leukemia virus (4, 6).
- XMRV-infected LNCaP cells were used to compare the 83A25 mAb with the anti-SFFV mAb for virus detection by indirect immunofluorescence (Fig. 1). The anti-SFFV mAb was chosen for comparison because it was used for XMRV detection in previous studies (1). Quantitative immunofluorescence analysis with a Zeiss Axioplan 2 microscope and Metamorph software indicated that mAb 83A25 had at least four-fold greater sensitivity for virus detection in comparison with the anti-SFFV mAb.
- XMRV-infected and uninfected LNCaP cells were formalin-fixed and paraffin-embedded as positive and negative controls for immunohistochemistry (IHC) analysis. 5 μm-sections were cut for the optimization of IHC conditions with mAb 83A25 (Fig. 2). FF-PE sections of these cells have been used as controls for IHC analysis of FF-PE tissues.
- 28 breast cancer and 5 normal breast FF-PE tissue samples that are de-identified were obtained from Asterand, Inc., Detroit, MI, for IHC analysis using our optimized protocol. Our results have indicated that 20% breast cancer specimens and none of the normal breast tissues expressed a protein that reacted with mAb 83A25 (Fig. 3).
- An amino acid search of the human genome for proteins that are closely related to different murine gammaretroviral Envs that are known to react with mAb 83A25 identified Env proteins of eight different class I human endogenous retroviruses (HERVs F(c)1, F(c)2, FRD, H, R(b), R/env3, T, and W) with sequence identities ranging from 43 to 51%.

REPORTABLE OUTCOMES: A XMRV-infected LNCaP cell line has been produced, and a monoclonal antibody has been identified for sensitive XMRV-detection by IHC. Optimum conditions for IHC detection of FF-PE XMRV-infected LNCaP cells have been developed. IHC analysis of de-identified FF-PE sections for 28 breast cancer and 5 normal breast tissues indicated that 20% of breast cancer specimens and none of the normal breast samples expressed a cellular protein that was detectable with mAb 83A25.

CONCLUSION: We have developed cell lines for detection of XMRV infection by immunohistochemistry and identified a monoclonal antibody that detects this retrovirus with greater sensitivity than that used in previous studies by other laboratories. Using this monoclonal antibody and cell lines as controls, we have optimized conditions for performing IHC analysis of FF-PE breast cancer tissues. These conditions were used to analyze 28 breast cancer specimens and 5 normal breast tissues. Our results indicate that 20% of breast cancer specimens and none of the normal breast samples express a cellular protein that is detectable

with mAb 83A25. An amino acid search of the human genome for proteins that are closely related to different murine gammaretroviral Envs that are known to react with mAb 83A25 identified Env proteins of eight different class I human endogenous retroviruses (HERVs F(c)1, F(c)2, FRD, H, R(b), R/env3, T, and W) with sequence identities ranging from 43 to 51%.
These observations, therefore, suggest that the cellular protein that we have detected is a class I HERV Env, which may be associated with breast cancer. Our studies have resulted in the detection of a potentially novel breast cancer-associated protein. Further work will be required to test this hypothesis.

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